Attorney Docket No: 13761-0726

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Dated: 11/12, 2003 Name of Person Certifying: Peggy Nichols
Printed Name: PEGGY NICHOLS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Stallcup, et al.	Assignee:	USC
Serial No:	09/464,377	Examiner:	Prouty, Rebecca
Filing Date:	December 15, 1999	Group Art Unit:	1652
Title:	REGULATION OF GENE EXPRESSION BY PROTEIN METHYLATION		

Commissioner for Patents
P.O. Box 1450
Arlington, VA 22313-1450

DECLARATION OF MICHAEL R. STALLCUP, DAGANG CHEN, HENG HONG AND DANA W. ASWAD UNDER 37 C.F.R. § 1.132 (*IN RE KATZ*)

Sir:

We, Michael R. Stallcup, Dagang Chen, Heng Hong and Dana W. Aswad, who are named as coinventors of the above-identified application, hereby declare as follows:

1. We are the co-authors of the publication attached to this Declaration as Exhibit A, Chen et al. (1999) "Regulation of Transcription by a Protein Methyltransferase" *Science* **284**:2174-2177. Therefore, this publication describes our own work. Our co-authors of this publication are Han Ma, Stephen S. Koh, Shih-Ming Huang, and Brandon T. Schurter.

2. We are the inventors of any subject matter disclosed in the *Science* article and claimed in the above patent application. The individuals identified as our co-authors in paragraph 1 were working under the direction and supervision of at least one of us. The work described in the *Science* publication that was contributed by the co-authors named in

paragraph 1 and not named as coinventors did not rise to the level of inventorship in the above-identified application.

3. We are the true inventors of the subject matter claimed in application U.S. Serial No. 09/464,377.

4. We further declare that all statements made herein of our own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 10 Nov 2003 — Michael R. Stallcup

Michael R. Stallcup

Date: _____ — _____

Dagang Chen

Date: _____ — _____

Heng Hong

Date: _____ — _____

Dana W. Aswad

Attorney Docket No: 13761-0726

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Date: _____ _____

Michael R. Stallcup

Date: 10/28/03 Dagang Chen

Dagang Chen

Date: _____ _____

Heng Hong

Date: _____ _____

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Date: _____ _____

Michael R. Stallcup

Date: _____ _____

Dagang Chen

Date: 10/29/2003 _____

Heng Hong

Date: _____ _____

Dana W. Aswad

Attorney Docket No: 13761-0726

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Heng Hong

Date: 11/3/03 _____

Dana W. Aswad

U.S.S.N: 09/464,377
Attorney Docket No: 13761-0726
Client Docket No: 2804

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25 June 1999, Volume 284, pp. 2174–2177

Science

Regulation of Transcription by a Protein Methyltransferase

Dagang Chen,[1] Han Ma,[1] Heng Hong,[1] Stephen S. Koh,[1] Shih-Ming Huang,[1] Brandon T. Schurter,[2] Dana W. Aswad,[2] Michael R. Stallcup[1]*

Regulation of Transcription by a Protein Methyltransferase

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The p160 family of coactivators, SRC-1, GRIP1/TIF2, and p/CIP, mediate transcriptional activation by nuclear hormone receptors. Coactivator-associated arginine methyltransferase 1 (CARM1), a previously unidentified protein that binds to the carboxyl-terminal region of p160 coactivators, enhanced transcriptional activation by nuclear receptors, but only when GRIP1 or SRC-1a was coexpressed. Thus, CARM1 functions as a secondary coactivator through its association with p160 coactivators. CARM1 can methylate histone H3 in vitro, and a mutation in the putative *S*-adenosylmethionine binding domain of CARM1 substantially reduced both methyltransferase and coactivator activities. Thus, coactivator-mediated methylation of proteins in the transcription machinery may contribute to transcriptional regulation.

Nuclear hormone receptors (NRs) are a related group of hormone-regulated transcriptional activators that includes the receptors for steroid and thyroid hormones, retinoic acid, and vitamin D (*1*). Transcriptional activation by NRs is mediated by the NR (or p160) coactivators, a family of three related 160-kD proteins that includes SRC-1, GRIP1/TIF2, and pCIP/RAC3/ACTR/AIB1/TRAM1 (*2*). Transcriptional coactivators locally modify chromatin structure and help to recruit an RNA polymerase II transcription initiation complex to the gene promoter (*2*, *3*). The COOH-terminal AF-2 activation functions of NRs bind to p160 coactivators at multiple NR box motifs containing the sequence LXXLL (where L is leucine and X is any amino acid), located in the central region of the p160 polypeptide (*2*) (Fig. 1). The NH_2-terminal AF-1 activation functions of some NRs bind the COOH-terminal region of p160 coactivators (*4*–*6*). The activating signal received from the DNA-bound NRs by the p160 coactivator is transmitted to the transcription machinery by activation domains AD1 and AD2 of the p160 coactivators. AD1 binds CREB binding protein (CBP) or the CBP-related protein p300, which help to activate transcription because they contain a histone acetyltransferase (HAT) activity to modify chromatin structure; CBP and p300 also associate with other coactivators like p/CAF, which also contains HAT activity, and bind components of the basal transcription machinery (*7*, *8*). We recently demonstrated that AD2, a second putative AD located in the COOH-terminal region of p160 proteins (*7*, *9*), plays an important role in p160 coactivator function (*6*). To understand the mechanism of downstream signaling by AD2, we sought to identify proteins that interact physically and functionally with this domain.

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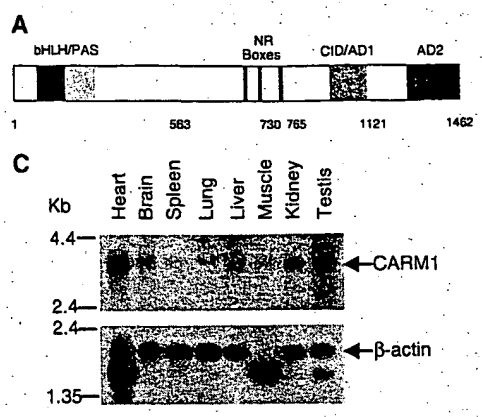

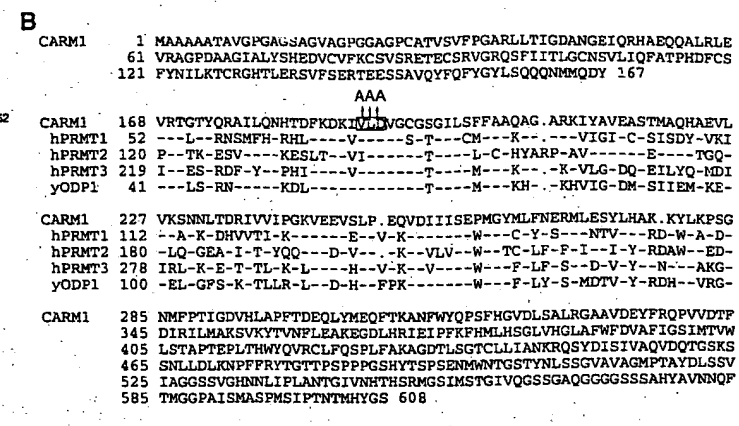

Fig. 1. (A) Functional domains of p160 coactivators. Domains of GRIP1 are shown (9). bHLH, basic-helix-loop-helix sequence; PAS, Per-Arnt-Sim domain (20); CID, CBP interaction domain; AD, activation domain; vertical bars, NR boxes (LXXLL sequences); numbers, position of GRIP1 amino acids. (B) CARM1 amino acid sequence, predicted from the cDNA sequence. The region of highest homology between CARM1, three other mammalian protein arginine methyltransferases, and one yeast protein arginine methyltransferase (13, 21) is aligned with dashes representing the same amino acid as in CARM1 and dots representing spaces inserted for optimum alignment. Amino acids 143 to 457 of CARM1 share 30% identity with hPRMT1 and yODP1. The location of a VLD-to-AAA substitution used in these studies is indicated. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (C) Expression of CARM1 mRNA in various mouse tissues was examined by hybridizing a 1.7-kb Bam HI–Bgl II cDNA fragment (representing CARM1 codons 346 to 608 and ~0.9 kb of 3′-untranslated region) to a multiple-tissue Northern (RNA) blot (Clontech) as described (22). A human β-actin cDNA probe was used as control. Positions of RNA size markers are shown on the left (in kilobases).

By using the yeast two-hybrid system to screen a mouse 17-day embryo cDNA library, we isolated a cDNA clone encoding a 608–amino acid protein that bound to COOH-terminal amino acids 1121 to 1462 of GRIP1 ($GRIP1_C$) (10). The central portion of the coding region has extensive homology to a family of proteins with arginine-specific protein methyltransferase activity (Fig. 1B). The protein, coactivator-associated arginine (R) methyltransferase 1 (CARM1), has a 3.8-kb mRNA that was widely but not evenly expressed in adult mouse tissues (Fig. 1C). CARM1, attached to agarose beads as a glutathione *S*-transferase (GST) fusion protein, bound a labeled COOH-terminal GRIP1 fragment synthesized in vitro, but did not bind protein fragments representing other parts of GRIP1 (Fig. 2A). GST-CARM1 bound all three members of the p160 coactivator family.

Because CARM1 is homologous to protein arginine methyltransferases (Fig. 1B), we tested it for methyltransferase activity. Protein arginine methyltransferases transfer a methyl group from *S*-adenosylmethionine to the guanidino group nitrogen atoms in arginine residues of specific proteins. In vitro protein substrates for these enzymes include histones and proteins involved in RNA metabolism such as hnRNPA1, fibrillarin, and nucleolin (11, 12). CARM1 preferentially methylated histone H3, either in a bulk histone preparation or individually purified form (Fig. 3). The related mammalian enzyme protein arginine (R) methyltransferase 1 (PRMT1) (13) preferentially methylated histone H4. Both enzymes also methylated individually purified histone H2A, but not H2A in an unfractionated histone preparation. Proteins and synthetic peptides containing arginine residues in glycine-rich regions, which were good substrates for PRMT1 (12, 13), were methylated very inefficiently by CARM1 (14).

Because CARM1 bound to the COOH-terminal region of GRIP1 containing the AD2 activation domain, we tested CARM1 for its ability to enhance the function of AD2. In transiently transfected CV-1 cells, $GRIP1_C$ (amino acids 1122 to 1462) was a relatively weak activation domain when fused with Gal4 DNA binding domain (DBD); coexpression of CARM1 enhanced the activity of Gal4DBD-$GRIP1_C$ by up to 10-fold but had no effect on the activity of Gal4DBD (Fig. 4A). The activity level depended on the amount of Gal4DBD-$GRIP1_C$ and CARM1 expression vectors transfected, and CARM1 expression had little if any effect on the activity of Gal4DBD fused to $GRIP1_{5-765}$ or $GRIP1_{563-1121}$, which contains AD1 (15). Thus, CARM1's coactivator function was specific for AD2 and correlated with its ability to bind $GRIP1_C$.

CARM1 also enhanced GRIP1's coactivator function for NRs. In transiently transfected mammalian cells, hormone-dependent activation of reporter genes by androgen receptor, estrogen receptor, or thyroid hormone receptor was enhanced 2- to 27-fold by coexpression of GRIP1 (Fig. 4B, columns a, b, and d). These activities were further enhanced two- to four-fold by coexpression of CARM1 with the NR and GRIP1 (column e), and all of this activity was hormone-dependent (column f). However, in the absence of exogenous GRIP1, CARM1 had little or no effect on the activity of the NR (column c). Similar results were observed when SRC-1a was substituted for GRIP1 (15). Thus, although GST-CARM1 bound weakly to some NRs in vitro (15), the fact that CARM1's ability to enhance NR activity depended on coexpression of exogenous GRIP1 is consistent with a model whereby CARM1's functionally important interaction with NRs is indirect, through a p160 coactivator. Expression of exogenous NRs presumably renders the levels of endogenous p160 coactivators limiting, so that the effects of exogenous CARM1 expression can only be observed when additional p160 coactivators are also expressed. Thus, CARM1 acts as a secondary coactivator for NRs by binding to and enhancing the activity of primary p160 coactivators.

The presence of both protein methyltransferase and transcriptional coactivator activities in CARM1 suggests that methylation of histones or other proteins, or both, may play a role in transcriptional regulation. As an initial test of this hypothesis, we mutated CARM1 cDNA to substitute alanines for three amino acids, valine 189, leucine 190, and aspartic acid 191, located in the highly conserved region (Fig. 1B) that is proposed to be important for *S*-adenosylmethionine binding and thus for methyltransferase activity (13). This mutation (VLD to AAA) com-

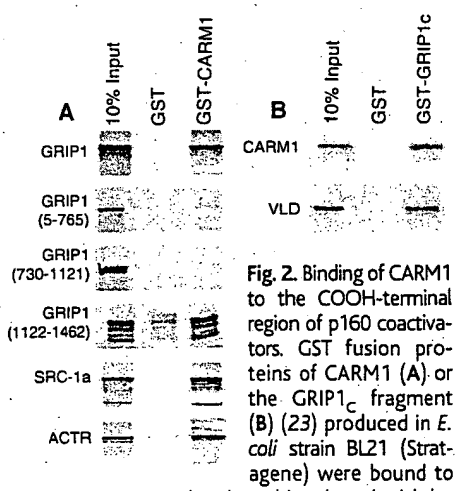

Fig. 2. Binding of CARM1 to the COOH-terminal region of p160 coactivators. GST fusion proteins of CARM1 (A) or the $GRIP1_C$ fragment (B) (23) produced in *E. coli* strain BL21 (Stratagene) were bound to glutathione-agarose beads and incubated with labeled proteins translated in vitro from pSG5 vectors encoding GRIP1, GRIP1 fragments, SRC-1a, CARM1, or the CARM1 VLD-to-AAA mutant (23), or from pCMX.ACTR (7); bound labeled proteins were eluted and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) as described (19).

pletely eliminated the ability of CARM1 methylate histone H3 in vitro (Fig. 3). The same mutation substantially reduced CARM1's ability to enhance transcriptional activation by Gal4DBD-GRIP1$_C$ (Fig. 4A) or by the estrogen receptor in the presence of GRIP1 (Fig. 4B, column g). However, the mutant and wild-type CARM1 were expressed at similar levels in transfected cells (*15*) and bound in vitro to GST-GRIP$_C$ at similar levels (Fig. 2B). The correlated loss of the methyltransferase and coactivator activities supports the hypothesis that CARM1's methyltransferase activity is important for its coactivator function.

Transcriptional coactivators are components in a signaling pathway that emanates from DNA-bound transcriptional activator proteins and results in local modification of chromatin structure and recruitment of a transcription initiation complex to the promoter of a specific gene. p160 coactivators receive the activating signal through direct contact with DNA-bound NRs (*2*, *4–6*) and transmit the signal onward through activation domains AD1 and AD2 (*6*, *7*, *9*). AD1 binds CBP or p300, which serve as secondary coactivators, at least in part by acetylating histones or other proteins, or both, in the transcription initiation complex (*7*, *9*, *16*). There is abundant evidence that modulation of chromatin structure by acetylation of histones plays an important role in transcriptional activation of specific genes and that histone deacetylation is connected with gene repression (*2*, *3*, *17*). Our data suggest that the AD2 domain of p160 coactivators propagates an activating signal through CARM1. The coactivator function of CARM1 correlated with CARM1's ability to bind the COOH-terminal domain of GRIP1 and with its methyltransferase activity. While the in vivo protein target of CARM1 methyltransferase is unknown, this coactivator's ability to methylate histone H3 in vitro suggests a possible role for histone methylation in transcriptional activation. In vivo methylation of histones on both lysine and arginine residues has been documented (*11*), and recent studies have indirectly suggested roles for protein methylation in other cellular processes, including RNA processing and receptor-mediated signaling (*11*, *18*). However, the specific methyltransferases, protein substrates, or roles played by methylation in these phenomena have not been determined. We propose that methylation of histone H3 or other proteins, or both, in the transcription initiation complex by CARM1 may cooperate with protein acetylation by other coactivators to remodel chromatin or otherwise activate transcription.

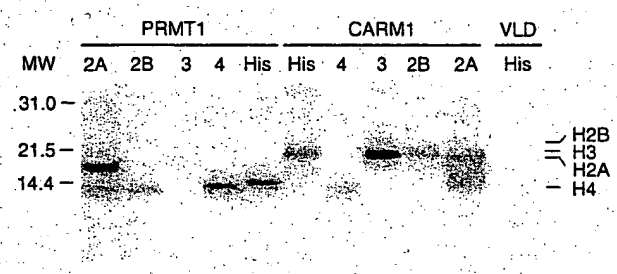

Fig. 3. Histone methyltransferase activity of CARM1. Calf thymus histones (Boehringer-Mannheim) were incubated for 30 min at 30°C in 32.5-μl reactions containing 20 mM tris-HCl, 0.2 M NaCl, 4 mM EDTA (pH 8.0); 0.32 mg/ml individual histone (lanes 2A, 2B, 3, and 4) or 1.3 mg/ml mixed histone (His); 0.020 to 0.037 mg/ml GST-CARM1, GST-CARM1 VLD-to-AAA mutant (VLD), or GST-PRMT1; and 7 μM *S*-adenosyl-L-[methyl-^{3}H]methionine (specific activity of 14.7 Ci/mmol). Reactions were stopped by addition of SDS-NuPAGE sample buffer (Novex), and 40% of each stopped reaction was then subjected to SDS-PAGE in 4 to 12% NuPAGE Bis-Tris gradient gels (Novex) with the Na-MES running buffer. Gels were stained with Coomassie blue R-150 to visualize histone bands and then subjected to fluorography (*24*) for 12 hours at −70°C on sensitized Kodak XAR-5 film. Sizes (in kilodaltons) and positions of molecular weight markers (MW) are shown at left, and positions of stained histone bands, on the right. Two different preparations of the GST-CARM1 VLD mutant failed to show detectable activity with any substrate.

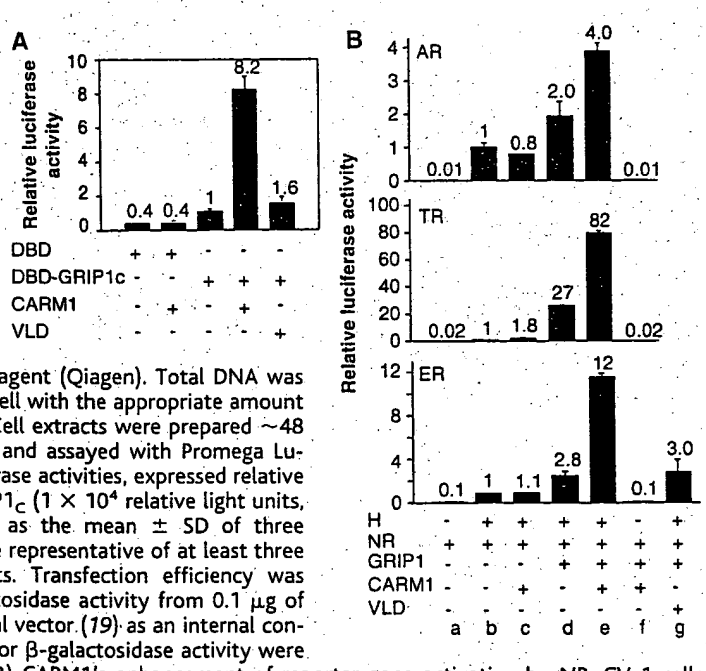

Fig. 4. (A) CARM1's enhancement of reporter gene activation by Gal4DBD-GRIP1$_C$. CV-1 cells in six-well dishes (3.3-cm diameter well) were transiently transfected with 0.5 μg of GK1 reporter gene (luciferase gene controlled by Gal4 binding sites) (*4*) and 0.5 μg of each of the other indicated vectors (*23*), with SuperFect Transcription Reagent (Qiagen). Total DNA was adjusted to 2.0 μg per well with the appropriate amount of empty pSG5 vector. Cell extracts were prepared ~48 hours after transfection and assayed with Promega Luciferase Assay kit. Luciferase activities, expressed relative to that of Gal4DBD-GRIP1$_C$ (1 × 10^4 relative light units, or RLU), are presented as the mean ± SD of three transfected wells and are representative of at least three independent experiments. Transfection efficiency was monitored with β-galactosidase activity from 0.1 μg of cotransfected pCMV-βgal vector (*19*) as an internal control, but no corrections for β-galactosidase activity were made. DBD, Gal4DBD. (B) CARM1's enhancement of reporter gene activation by NR. CV-1 cells were transiently transfected as in (A) with the following vectors, as indicated: 0.5 μg of NR expression vector pSVAR$_0$ (*25*) expressing androgen receptor (AR), pHE0 (*26*) expressing estrogen receptor (ER), or pCMX.hTRβ1 (*27*) expressing thyroid hormone receptor β1 (TR); 0.5 μg of a luciferase reporter gene with an appropriate promoter, MMTV promoter for AR, or MMTV promoter with the native glucocorticoid response elements replaced by a single estrogen response element for ER, or palindromic thyroid hormone response element for TR (*28*); 0.5 μg of pSG5.HA-GRIP1; and 0.5 μg of the wild-type or the VLD-to-AAA mutant of pSG5.HA-CARM1. After transfection, cells were grown in charcoal-treated serum; where indicated, 20 nM hormone (H), that is, dihydrotestosterone for AR, estradiol for ER, or triiodothyronine for TR, was included during the last 40 hours of culture. Luciferase activities, expressed relative to those of NR plus hormone (1 × 10^6 RLU for AR, 2 × 10^5 RLU for TR, and 3 × 10^4 RLU for ER), are presented as the mean ± SD of three transfected wells and are representative of at least six independent experiments. In six experiments the enhancement caused by the CARM1 mutant was 15 ± 9% (SE) of that caused by wild-type CARM1.

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23. Plasmid construction. Mammalian cell expression vectors: pSG5.HA was constructed by inserting a synthetic sequence coding for a translation start signal, hemagglutinin A (HA) tag, Eco RI site, and Xho I site into the Eco RI–Bam HI site of pSG5 (Stratagene), which has SV40 and T7 promoters. The original Eco RI site was destroyed by this insertion, but the Bam HI site was preserved, leaving a multiple cloning site after the HA tag containing Eco RI, Xho I, Bam HI, and Bgl II sites. The following protein-coding regions were cloned into pSG5.HA, in frame with the HA tag, with the indicated insertion sites: GRIP1$_{5-1462}$ (full length) and CARM1$_{3-608}$ (full length) at the Eco RI site; GRIP1$_{5-765}$ at the Eco RI–Xho I site; GRIP1$_{730-1121}$ and GRIP1$_{1121-1462}$ were Eco RI–Sal I fragments inserted at the Eco RI–Xho I site; SRC-1a$_{1-1441}$ (full length) was a Sma I–Sal I fragment inserted at the Eco RI site, which was blunted by filling with Klenow polymerase, and the Xho I site. Expression vectors for Gal4DBD fused to various GRIP1 fragments were constructed by inserting the appropriate fragments into pM (Clontech) as follows: GRIP1$_{1122-1462}$, Eco RI–Bgl II fragment inserted into Eco RI–Bam HI site; GRIP1$_{563-1121}$ and GRIP1$_{5-765}$, Eco RI–Sal I fragments inserted into homologous site. Vectors for GST fusion proteins were constructed in pGEX-4T1 (Pharmacia): for GST-CARM1 the original 3.2-kb Eco RI fragment from pGAD10.CARM1 was inserted; for GST-GRIP1$_C$ (amino acids 1122 to 1462) an Eco RI–Sal I fragment was inserted. The CARM1 VLD-to-AAA mutation was engineered with the Promega Gene Editor Kit.
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